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TOXICITY OF NITRO-HETEROCYCLIC AND NITROAROMATIC ENERGETIC MATERIALS TO ENCHYTRAEID WORM *Enchytraeus crypticus* IN A NATURAL SANDY LOAM SOIL

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U.S. Environmental Protection Agency is developing Ecological Soil Screening Level (Eco-SSL) values for ecological risk assessment of contaminants at Superfund sites. Insufficient information for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB to generate Eco-SSLs necessitated standardized toxicity testing to fill the data gaps. The Enchytraeid Reproduction Test (ISO/16387:2001) was adapted using *Enchytraeus crypticus* in these studies. Tests were conducted in Sassafras sandy loam soil, which supports relatively high bioavailability of energetic materials. Weathering/aging procedures for amended soil were incorporated in the study to better reflect the exposure conditions in the field soils. Definitive toxicity tests conducted with both freshly amended and weathered/aged amended soils showed that EM toxicity order based on EC_{20} values for juvenile production in tests with *E. crypticus* was TNB > 2,4-DNT > 2,6-DNT > RDX with EC_{20} values of 45, 116, 194, and 585 mg kg^{-1}, respectively. The octahydro-1, 3, 5, 7 - tetranitro-1, 3, 5, 7 - tetrazocine (HMX) did not adversely affect adult survival or juvenile production up to 21750 mg kg^{-1} treatment. These study results will be provided to the Eco-SSL workgroup for review and inclusion in the Eco-SSL database, and for developing Eco-SSLs for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.

15. SUBJECT TERMS

RDX	TNB	*Enchytraeus crypticus*
HMX	Toxicity Assessment	Natural soil
2,4-DNT	Weathering/aging	Bioavailability
2,6-DNT	Ecological Soil Screening Level	

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PREFACE

The work described in this report was authorized under Project No. SERDP CU-1221. The work was started in April 2001 and completed in July 2003.

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1. INTRODUCTION

Many sites associated with military operations that involve munition manufacturing, disposal, testing, and training contain elevated levels of explosives and related materials in soil. Concentrations of explosives in soil have been reported to exceed 87,000 mg kg^{-1} for TNT and 3,000 mg kg^{-1} for RDX and HMX (Simini *et al.*, 1995). Although the energetic materials (EM) RDX and HMX are persistent and highly mobile in the environment, their effects on soil biota have not been sufficiently investigated. Scientifically based ecological soil screening levels (Eco-SSLs) are needed to identify contaminant explosive levels in soil that present an acceptable ecological risk. To address this problem, the U.S. Environmental Protection Agency (USEPA) in conjunction with stakeholders is developing Eco-SSLs for contaminants frequently found at Superfund sites. Eco-SSLs are defined as concentrations of chemicals in soil that, when not exceeded, will be protective of terrestrial ecosystems from unacceptable harmful effects. These Eco-SSL concentrations can be used in a Screening Level Ecological Risk Assessment (ERA) to identify those contaminants in soil that warrant additional evaluation in a Baseline ERA, and to eliminate those that do not. Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species relevant to soil ecosystems. The Eco-SSL workgroup, after an extensive literature review (USEPA, 2000), determined that there was insufficient information for explosives to generate Eco-SSLs for soil invertebrates, which necessitated our study to fill this knowledge gap.

This study was designed to produce benchmark data for the development of Eco-SSLs for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) for soil invertebrates, and meet specific criteria (USEPA, 2000), including: (1) tests were conducted in soil having physico-chemical characteristics that support relatively high bioavailability of chemicals; (2) experimental designs for laboratory studies were documented and appropriate; (3) both nominal and analytically determined concentrations of chemicals of interest were reported; (4) tests included both negative and positive controls; (5) chronic or life cycle tests were used; (6) appropriate chemical dosing procedures were reported; (7) concentration-response relationships were reported; (8) statistical tests used to calculate the benchmark and level of significance were described; and (9) the origin of test species were specified and appropriate.

Several soil invertebrate toxicity tests, for which standardized protocols have been developed, could be used effectively to assess the toxicity and to derive protective benchmark values for energetic materials (Stephenson *et al.*, 2002; Løkke and Van Gestel, 1998). We adapted the Enchytraeid Reproduction Test (ISO/16387: 2001) for use in these studies. This bioassay was selected on the basis of its ability to measure chemical toxicity to ecologically relevant test species during chronic assays, and its inclusion of at least one reproductive

component among the measurement endpoints. The primary objective of these studies was to quantify EM toxicities to the soil invertebrate *Enchytraeus crypticus* for production of benchmark data that can be used in development of Eco-SSLs for explosive contaminants in soil. The Enchytraeid Reproduction Test was specifically modified to comply with Eco-SSL testing conditions.

2. MATERIAL AND METHODS

2.1 Test Soil.

A natural soil, Sassafras sandy loam [Fine-loamy, siliceous, mesic Typic Hapludult] (SSL) was used in this study to assess the toxicity of test chemicals to *E. crypticus*. This soil was selected for developing ecotoxicological values protective of soil biota because it has physical and chemical characteristics supporting relatively high bioavailability of the test chemicals (low organic matter and clay contents). The SSL soil was collected from an open grassland field on the property of the U.S. Army Aberdeen Proving Ground (APG; Edgewood, MD). Vegetation and the organic horizon were removed to just below the root zone and the top six inches of the A horizon were then collected. The soil was sieved through a 5-mm^2 mesh screen, air-dried for at least 72 hours and mixed periodically to ensure uniform drying, passed through a 2-mm sieve, then stored at room temperature before use in testing. Soil was analyzed for physical and chemical characteristics by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Results of these analyses are presented in Table 1.

Table 1. Physical and chemical characteristics of Sassafras sandy loam soil analyzed by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD.

Soil Parameter	Sassafras Sandy Loam
Sand %	69
Silt %	13
Clay %	17
Texture	Sandy loam
CEC cmol kg^{-1}	5.5
Organic matter %	1.2
pH	5.2

2.2 Test Chemicals.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; CAS: 121-82-4; Purity: 99%), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; CAS: 2691-41-0; Purity: 99%), 2,4-dinitrotoluene (2,4-DNT; CAS: 121-14-2; Purity: 97%), 2,6-dinitrotoluene (2,6-DNT; CAS: 606-20-2; Purity: 98%), and 1,3,5-trinitrobenzene (TNB; CAS: 99-35-4; Purity: 99.7%) were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada). Beryllium sulfate ($BeSO_4 \cdot 4H_2O$; CAS: 7787; Purity: 99.99%) was used as the positive control in these tests. Acetone (CAS: 67-64-1; HPLC Grade) was used for preparing EM solutions during soil amendments. Acetonitrile (CAS: 75-05-8; HPLC Grade) was used for extractions for chemical analyses. Methanol (CAS: 67-56-1, Chromatography grade, Purity: 99.9%) was used in determinations by HPLC. Certified standards of the energetics (AccuStandard, Inc., New Haven, CT) were used during HPLC determinations. Unless otherwise specified, ASTM type I water (American Society of Testing and Materials, http://www.astm.org) obtained using Milli-RO® 10 Plus followed by Milli-Q® PF Plus systems (Millipore®, Bedford, MA) was used throughout the studies. Glassware was washed with phosphate-free detergent, followed by rinses with tap water, ASTM type II water, analytical reagent grade nitric acid 1% (v/v), then with ASTM type I water.

2.3 Soil Amendment Procedures.

Sassafras sandy loam soil was individually amended with RDX, HMX, 2,4-DNT, 2,6-DNT or TNB. Each treatment concentration of EM for range-finding tests was prepared separately in glass volumetric flasks and dissolved in acetone. This was necessary to dissolve the nonpolar chemicals, giving a more homogeneous mixture than the addition of solid chemical crystals to soil. Soil was spread to a thickness of 2.5 cm. The EM/acetone solution was pipetted evenly across the soil surface, ensuring that the volume of solution added at any one time did not exceed 15% (v m^{-1}) of the dry mass soil. After addition of the EM solution, the volumetric flask was rinsed twice with a known volume of acetone and pipetted onto the soil. If the acetonitrile-extractable volume of solution needed to amend the soil exceeded 15% (v m^{-1}), the solution was added in successive stages, allowing the acetone to evaporate for a minimum of 2 hours under a chemical hood. The same total EM/acetone solution volume at different EM concentrations was added to every treatment, equating the volume required to dissolve the EM at the highest concentration tested. Amended soil was then air-dried overnight (minimum of 18 hours) in a dark chemical hood to prevent photolysis of the EM. Each amended soil sample was transferred into a fluorocarbon-coated high-density polyethylene container and mixed for 18 hours on a three-dimensional rotary mixer. Initial concentrations of EMs for toxicity tests were prepared by adding test chemicals into an aliquot of SSL soil. The final nominal target treatment concentrations for definitive tests with 2,4-DNT; 2,6-DNT; or TNB were prepared by mixing initially prepared soil amended with the appropriate EM with clean SSL soil for 18 hours on a three-dimensional rotary mixer. Treatment concentrations of RDX or HMX were prepared individually using direct amendments of EM/acetone mixtures to achieve nominal target concentrations. The exceptions were 10,000 and 20,000 mg kg^{-1} treatments, which exceeded solubility levels of RDX or HMX in acetone carrier. These were prepared by adding appropriate amounts of dry crystalline RDX or HMX to clean SSL soil. The same acetone volume was

added to every RDX or HMX concentration treatment. Carrier controls were treated with the carrier solvent only. After three-dimensional mixing, soil was hydrated with ASTM type I water to 100% of the soil water holding capacity (WHC; 18% water, on a the basis of the dry soil mass) for toxicity testing, or 60% of the WHC for the weathering/aging procedure. Hydrated soil prepared for toxicity tests was allowed to equilibrate for 24 hours before exposing potworms.

2.4 Measurement of Soil pH.

The pH of the test soils was determined at the beginning of each definitive toxicity test using a method adapted from the Soil Survey Laboratory Methods Manual (USDA, 1996). The pH electrode was rinsed thoroughly with ASTM type I water, blotted dry, standardized with pH 4 and pH 7 buffers, rinsed and blotted. Five grams of ASTM type I water was added to 5 g soil. The soil slurry was Vortexed for 10 seconds every five minutes for 30 minutes. The soil slurry was then Vortexed for 10 seconds one minute before pH measurement. The pH was measured in the solution above the soil surface while stirring gently until the reading stabilized. The electrode was rinsed with ASTM type I water and blotted.

2.5 Treatment Concentrations.

2.5.1 Range-Finding Tests.

Range-finding tests were conducted with freshly amended soils to determine treatment concentrations for definitive tests. Soils used in range-finding tests were amended with nominal RDX or HMX concentrations of 10, 100, 500, 1000, 5000 and 10000 mg kg^{-1}. Nominal EM test concentrations selected for the range-finding tests with 2,4-DNT and 2,6-DNT were 0, 10, 50, 100, 500, and 1,000 mg kg^{-1}. Concentrations selected for the range-finding test with TNB were 0, 10, 50, 100, 500, 1000, 5000, and 10000 mg kg^{-1}.

2.5.2 Definitive Tests.

Data from the range finding tests were used to determine the treatment concentrations for definitive tests. Definitive tests to assess the independent effects of EMs were conducted in freshly amended and weathered/aged amended SSL soil. Nominal RDX or HMX concentrations selected for the definitive tests in freshly amended soil included 0, 300, 600, 1200, 2400, 4800, 10000, 20000 mg kg^{-1}, and 0, 300, 600, 1200, 2500, 5000, 10000, 20000 mg kg^{-1}, respectively. Nominal concentrations selected for the definitive tests in SSL soil freshly amended with 2,4-DNT or 2,6-DNT were 0, 2, 4, 8, 12, 24, 48, 64, and 80 mg kg^{-1}. Nominal concentrations selected for the definitive test with TNB freshly amended soil were 0, 4, 8, 16, 32, 64, 128, 256, and 384 mg kg^{-1}. Nominal test chemical concentrations selected for the definitive tests in weathered/aged amended SSL soil were:

RDX (mg kg^{-1}) 0, 1200, 2400, 4800, 10000, 20000
2,4-DNT (mg kg^{-1}) 0, 8, 12, 24, 48, 64, 80, 160, 320
2,6-DNT (mg kg^{-1}) 0, 24, 48, 64, 80, 160, 320
TNB (mg kg^{-1}) 0, 16, 32, 64, 128, 256, 384

Limit test was conducted with weathered/aged HMX amended SSL soil using 0 and 20000 mg kg^{-1} treatments. All definitive tests included carrier (acetone) controls and positive controls. Positive controls were prepared as solution of beryllium sulfate in ASTM type I water using 45 mg kg^{-1} Be nominal concentrations in all tests with 2,4-DNT, 2,6-DNT and TNB. Nominal beryllium concentration 50 or 47 mg kg^{-1} was used in freshly amended or weathered/aged amended SSL soil, respectively in tests with RDX or HMX. Nominal test concentrations of all energetic compounds were verified using USEPA Method 8330 (USEPA, 1998).

2.6 Weathering/Aging of Amended Soil.

Standardized methods for weathering/aging of explosives in soil are not available. We have developed approaches that simulate, at least partially, the weathering and aging process in soil and more closely approximate the exposure effects on soil biota in the field. This included exposing both treated and control soils, initially hydrated to 60 percent of the WHC, in open Teflon®-coated chemically inert containers in the green house to alternating wetting and drying cycles for three months. All soil treatments were weighed and readjusted to their initial mass by adding ASTM type I water twice each week. All soil treatments were brought to 100% of the WHC (18% water, on the basis of the dry soil mass) 24 hours prior to commencement of toxicity tests for initiation of bioassays. The effect of weathering and aging on EM ecotoxicity was determined by comparing test results in weathered/aged amended soils with those obtained using freshly amended soils.

2.7 Chemical Extractions and Analyses.

Acetonitrile extractions of soils were performed according to USEPA Method 8330 at the beginning of each definitive test, using freshly amended or weathered/aged amended soils, respectively. Samples for chemical analysis were taken after the 24-h hydration. For each treatment, 2.0 g soil was weighed in triplicate into 50-mL polypropylene centrifuge tubes, 10 mL acetonitrile was added and the samples vortexed for 1 min, then sonicated in the dark for 18 hours at 20°C. Five mL of sonicated sample were transferred to a glass tube, to which 5 mL of $CaCl_2$ solution (5 g L^{-1}) was added. Supernatant was filtered through 0.45 µm PTFE syringe cartridges. Soil extracts were analyzed and quantified using an HPLC. In this report, acetonitrile soil extraction is reported as the concentration in dry soil.

In addition to acetonitrile extraction, soil samples were extracted using an Adapted Toxicity Characteristic Leaching Procedure (ATCLP; Haley *et al.*, 1993) at the beginning of each definitive test with freshly amended or weathered/aged amended soils. The ATCLP is based on modification of the Toxicity Characteristic Leaching Procedure (TCLP) (40 CFR Part 268.41, Hazardous Waste Management, Method 1311). The modification involved substitution of CO_2-saturated ASTM type I water for acetic acid, better simulating soil-water conditions due to respiration by soil biota. Prior to ATCLP extraction, soil samples were equilibrated in the dark for 24 h at room temperature, after addition of ASTM type I water (60% of WHC). All analytical measurements were done in triplicate at the beginning of each test. For

each treatment concentration, 4g of soil was transferred into 20 mL vials. Sixteen mL of CO_2-saturated water at pH 4.0 was added to the vials. Then the vials were rapidly sealed tight. Soil samples were vortexed 45 sec, then mixed in the dark for 18 hours using a rotary mixer (30 rpm) at room temperature. Soil solids were allowed to settle; then, supernatants were filtered through 0.45 µm PTFE syringe cartridges. An equivalent volume of acetonitrile was added to filtered soil extract prior to HPLC analysis. In this report, ATCLP soil extraction is referred to as the water-soluble fraction of EM.

The soil extracts were analyzed by reversed-phase HPLC using a modified EPA Method 8330. The method was modified in two ways. First, the final solvent for the energetic compounds was a mixture of 60 parts water and 40 parts acetonitrile rather than a 50:50 ratio. Secondly, the flow rate of the 50:50 methanol:water mobile phase was 1.0 ml/min rather than 1.5 ml/min as the method calls for. A 25 cm x 4.6 mm x 5 micron particle size C-18 column was used for all determinations since only one energetic compound was analyzed at a time. The instrument used was a Beckman *System Gold*, consisting of a model 126 programmable solvent module, model 168 diode array detector and a model 507 automatic sampler. Calibration curves were generated before each HPLC run by dissolving certified standards (AccuStandard, Inc., New Haven, CT) of RDX and HMX in 60:40 water:acetonitrile in a range of concentrations appropriate for each run. The method detection limit was 0.05 mg kg^{-1}. Blanks and standards were placed intermittently between unknown samples to maintain quality assurance of the samples. All reagents used in extraction of chemicals from soils were either reagent or trace metal grade, and ASTM Type I water was used throughout the analytical studies. Glassware was washed with phosphate-free detergent followed by rinses with tap water, ASTM type II water, nitric acid 1% (v/v) and, again with ASTM type I water. Nominal and determined (measured) concentrations used in the definitive tests are shown in Tables 2 through 10.

2.8 Toxicity Assessment.

The Enchytraeid Reproduction Test (ERT) was used to assess the effects of EMs on the reproduction of the enchytraeid worm *Enchytraeus crypticus*. The test is an adaptation of an International Standardization Organization (ISO) bioassay ISO/16387 *Soil quality — Effects of pollutants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction and survival* (ISO, 2001). The ERT is a Chronic/Life-Cycle Assay. The ISO Guideline for this assay was originally developed for use with Artificial Soil (USEPA Standard Artificial Soil), however our research showed that this test could also be conducted using natural soils (Kuperman *et al.*, 1999; 2004). The ISO ERT was initially developed using the enchytraeid worm species *Enchytraeus albidus*. Results of our previous studies using *E. albidus* showed that this species requires soils containing high organic matter content with a soil pH 6 (±0.5) for optimal test conditions. This species performed poorly in natural soils with physical and chemical characteristics that support a higher level of EM bioavailability (Kuperman *et al.*, 1999). The species of Enchytraeidae, *E. crypticus*, listed in the ISO protocol as an acceptable alternative to *E. albidus*, was selected for toxicity testing.

2.8.1 Principle of the Test.

Adult *E. crypticus* are exposed to a range of concentrations of the test chemical added to soil. The test consists of two steps. They are a range-finding test in which adult survival and total number of juveniles produced are assessed using few treatment concentrations (five) and reduced number of replicates (two), and a definitive test in which the same endpoints are assessed using greater number of concentrations and replicates. The duration of each test is four weeks. After the first two weeks, the adult worms are removed, counted, and any morphological changes are recorded. After an additional two-week exposure, the number of juveniles produced is counted. The number of adults and juveniles in treatment concentrations are compared to numbers in the control(s) to quantify ecotoxicological parameters. These parameters include the bounded No Observed Effect Concentration (NOEC), the bounded Lowest Observed Effect Concentration (LOEC) and the effective concentration that causes a p percent reduction in juvenile numbers, ECp (e.g., EC_{20}, and EC_{50}).

2.8.2 Test Validity Criteria.

The validity criteria are included in the test as part of the Quality Control procedures. They include the following performance parameters for the negative controls:

1) The adult mortality does not exceed 20% after 14 days, in the range-finding and definitive tests

2) The average number of juvenile potworms per test container at the end of the test is greater than 2.5x the initial number of adult potworms per test container

3) The coefficient of variation for the mean number of juveniles is ≤50% at the end of the test

2.8.3 Culturing Conditions.

Enchytraeid potworms were bred in 4.3-L clear plastic boxes (34 x 20 x 10 cm) filled with 2 kg (dry mass) SSL soil. The culture was kept in an incubator at 22 ± 1°C with continuous light. Soil moisture level was adjusted to 100% of WHC, and was maintained by periodic (once per week) mass checks and water adjustments. Soil in the breeding culture was aerated by carefully mixing it once per week.

The potworms were fed approximately twice a week with ground oats spread on the soil surface. If food from the previous feeding date remained on the soil surface, the amount of food given was adjusted. Every 2-3 months, the worms were transferred into a freshly prepared culture substrate.

Culturing conditions were regarded satisfactory if:

- Worms did not try to leave soil
- They moved quickly through the soil
- They exhibited a shiny outer surface without soil particles clinging to it

- They were whitish in color
- Worms of different ages were present

The potworm culture was considered healthy if worms reproduced continuously.

2.8.4 Test Performance.

Glass test containers (42 mm ID; 45 mm deep) were rinsed with acetone, tap water, and ASTM type I water before the test. Twenty grams of prepared soil hydrated to 100% of the WHC were added to each test container and 0.05 g of grounded oats were mixed with soil. The mass of each container with soil was recorded. Each treatment and controls were replicated four times for definitive tests (two for range-finding tests). Limit test with weathered/aged HMX amended SSL soil included eight replicates of treatment soils and four replicates of negative or positive controls.

Enchytraeid adult potworms with eggs in the clitellum region were collected from culture established in the same soil type (SSL) as soil used in the test. The selected worms were placed in a petri dish filled with a small amount of ASTM type I water for examination using a stereomicroscope. Worms with no eggs were discarded. Any invertebrates living in the cultures (such as mites) were also removed. Ten enchytraeid worms selected for uniformity (approximately 1 cm in length) were placed on top of prepared soil in each test container. Plastic wrap was stretched over the top of each container and secured with a rubber band. Three pinholes were made in the plastic wrap to facilitate air exchange. All containers were placed in an environment-controlled incubator at 22 ± 1°C, 16 h photoperiod. The containers were weighed once a week and the mass loss was replenished with the appropriate amount of ASTM type I water. Ground oats (0.05 g) were added to each test container at that time.

After two weeks, soil in each test container was carefully searched and adult potworms were removed and counted. Potworms were examined for any morphological or behavioral changes. The remaining test substrate, including any cocoons laid during the first two weeks of the test, was incubated for additional two weeks. After four weeks from the start of the test, soil in the test containers was fixed with 70% ethanol, and seven drops of Rosebengal biological stain (1% solution in ethanol) was added. Staining continued for minimum of 24 hours. The content of each test container was wet-sieved using a No. 100 (150 μm) mesh sieve and retained contents transferred to a counting tray where potworms were counted. Measurement endpoints included number of surviving adults after 14 days and number of juveniles produced after 28 days.

2.9 Data Analysis.

Juvenile production data were analyzed using nonlinear regression models described in Stephenson *et al.* (2000) and Kuperman *et al.* (2004). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select potential models. The logistic (Gompertz) model [1] had the best fit for data in all toxicity

tests except the test with SSL soil freshly amended with TNB where the logistic hormetic model [2] with an additional parameter to accommodate hormesis had the best fit for the data. The best fit of the lines generated by these models were closest to the data points, the variances were the smallest, and the residuals had the best appearance (i.e., most random scattering). These models were:

$$Y = a \times e([\log(1-p)] \times [C/\mathrm{ECp}]^b) \quad [1]$$

$$Y = (t \times [1 + hC] / \{1 + [(p + h\,\mathrm{ECp}) / (1 - p)] \times [C/\mathrm{ECp}]^b\} \quad [2]$$

where Y is the number of juveniles produced, a is the control response, e is the base of the natural logarithm, p is the percent inhibition/100 (e.g., 0.50 for EC_{50}), C is the exposure concentration in test soil, ECp is the estimate of effect concentration for a specified percent effect, t is the control response in the hormetic model, h is the hormetic effect parameter, and b is the scale parameter. The ECp parameters used in this study included the EM concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} parameter based on a reproduction endpoint is the preferred parameter for deriving soil invertebrate Eco-SSL values. The EC_{50}, a commonly reported value, and survival data were included to enable comparisons of the results produced in this study with results reported by other researchers. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for adult survival or juvenile production data. Mean separations were done using Fisher's Least Significant Difference (LSD) pairwise comparison tests. Student's t-Test was used in the limit test with weathered/aged HMX amended soil. A significance level of $p < 0.05$ was accepted for determining the NOEC and LOEC values. When NOAEC (bounded no observed adverse effect concentration) or LOAEC (bounded lowest observed adverse effect concentration) values were determined, the same statistical methods were used. All analyses were done using measured EM concentrations. Statistical analyses were performed using SYSTAT 7.0.1 (SPSS, 1997).

3. RESULTS

3.1 Analytical Determinations of Energetic Materials in Soil.

Concentrations of EMs in amended soils were determined at the beginning of each definitive toxicity test using both acetonitrile and ATCLP extractions. Results of these analyses are shown in Tables 2 through 10. Measured acetonitrile-extractable RDX concentrations in freshly amended soils averaged 101 (range: 92-109) percent of nominal concentrations. Measured RDX ATCLP-extractable concentrations averaged 9.6 (range: 0.4-34) percent of acetonitrile-extractable concentrations due to low solubility of RDX in water (Table 2). Measured RDX ATCLP-extractable concentrations in weathered/aged amended soils averaged 3 (range: 0.5-8.0) percent of acetonitrile-extractable concentrations (Table 3).

Table 2. Nominal and measured (mean, n = 3) RDX concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
300	304	19	101	102	6.4	33.7
600	656	19	109	95	18.3	14.5
1200	1194	22	100	115	3.38	9.6
2400	2203	72	92	115	25.1	5.2
4800	4558	143	95	123	2.9	2.7
10000	10062	366	101	76	0.8	0.8
20000	21383	1205	107	107	15.6	0.4

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 3. Nominal and measured (mean, n = 3) RDX concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
1200	1048	20	87	84	0.6	8.0
2400	2379	41	99	87	1.1	3.6
4800	3985	43	83	86	3.4	2.2
10000	9549	371	96	89	2.1	0.9
20000	18347	518	92	89	1.0	0.5

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 4. Nominal and measured (mean, n = 3) HMX concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
300	348	10	116	12.3	0.2	3.5
600	642	9	107	12.5	0.2	1.9
1200	1491	63	124	12.9	0.3	0.9
2500	2211	119	88	12.6	0.5	0.6
5000	5785	183	115	12.5	0.1	0.2
10000	10586	273	106	12.0	0.3	0.1
20000	21750	496	109	12.6	0.1	0.1

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 5. Nominal and measured (mean, n = 3) 2,4-DNT concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
2	0.95	0.2	48	0.8	0.001	84
4	3.0	0.3	74	1.3	0.01	45
8	6.5	0.4	81	2.4	0.05	37
12	9.9	0.5	82	5.0	0.01	50
24	20.3	0.3	85	3.8	0.04	19
48	40.9	2.6	85	8.1	0.08	20
64	55.0	0.5	86	33.4	0.22	61
80	64.7	1.5	81	43.4	0.09	67

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 6. Nominal and measured (mean, n = 3) 2,4-DNT concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
8	3	0.5	37	1.7	0.03	56
12	5	0.2	43	2.4	0.06	47
24	11	0.2	48	5.2	0.02	46
48	22	0.3	45	11.8	0.12	55
64	31	0.8	48	15.4	0.15	50
80	37	0.8	47	20.5	0.37	55
160	72	2.3	45	46.1	0.37	64
320	179	8.4	56	125	2.00	70

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 7. Nominal and measured (mean, n = 3) 2,6-DNT concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
2	5.3	0.1	267	1.4	0.01	27
4	7.7	0.9	191	2.2	0.01	28
8	9.4	0.3	117	3.8	0.01	40
12	12.9	0.2	108	5.8	0.04	45
24	20.0	0.8	83	10.6	0.08	53
48	40.2	2.0	84	24.8	0.04	62
64	51.1	1.0	80	32.9	0.12	65
80	64.0	1.6	80	40.5	0.11	63

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 8. Nominal and measured (mean, n = 3) 2,6-DNT concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
24	3.7	0.08	15	1.5	0.06	40
48	9.5	0.12	20	4.3	0.09	45
64	13.9	0.12	22	6.6	0.08	48
80	18.1	0.20	23	9.6	0.36	53
160	37.4	0.98	23	17.4	3.27	47
320	108.3	1.45	34	66.9	2.22	62

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Table 9. Nominal and measured (mean, n = 3) TNB concentrations (mg kg^{-1}) in freshly amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
4	2.3	0.08	58	BDL	BDL	BDL
8	2.6	0.1	32	0.043*	0.043*	1.7*
16	3.9	0.5	25	2.4	0.3	62
32	13.6	1.1	43	7.7	0.2	56
64	45	1.8	70	30.2	0.5	67
128	107	2.5	84	83.7	1.3	78
256	221	12.7	86	190.9	1.4	86
384	385	21.1	100	328.3	14.8	85

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.
* TNB was recovered in one (0.13 mg kg^{-1}) out of three replicates producing an average ATCLP extractable value of 0.043 mg kg^{-1}.

Table 10. Nominal and measured (mean, n = 3) TNB concentrations (mg kg^{-1}) in weathered/aged amended Sassafras sandy loam soil used in the toxicity tests with *E. crypticus*. Measured concentrations include acetonitrile-extractable (USEPA Method 8330) and water-extractable (Adapted Toxicity Characteristic Leaching Procedure, ATCLP) concentration values.

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/ Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
16	0.6	0.07	4	0.14	0.01	25
32	1.3	0.15	4	0.24	0.01	19
64	8.8	0.38	14	3.35	0.33	38
128	75.8	0.27	59	55.80	1.89	74
256	176	5.67	69	143.40	2.15	81
384	305	7.84	79	284.38	7.50	93

Table notes:
BDL - Below detection limit. Method Detection Limit, MDL = 0.05 mg L^{-1}; 0.5 mg kg^{-1} soil.

Weathering/aging of amended soils decreased acetonitrile-extractable RDX concentrations, on average, by 7 percent compared with acetonitrile-extractable concentrations in freshly amended soils (Table 3). ATCLP-extractable RDX concentrations decreased, on average, by 16 percent compared with freshly amended soils.

Measured HMX acetonitrile-extractable concentrations in freshly amended soils averaged 111 (range: 88-124) percent of nominal concentrations. Measured HMX ATCLP-extractable concentrations remained relatively stable and averaged one (range: 0.1-3.5) percent of acetonitrile-extractable concentrations (Table 4).

Weathering/aging of HMX amended soil decreased acetonitrile-extractable HMX concentration by 20 percent from 21750 to 17498 mg kg^{-1} in the single treatment used in the limit test. Measured HMX ATCLP-extractable concentration in weathered/aged soil was 18 mg kg^{-1} (0.1% of acetonitrile-extractable concentration). The ATCLP-extractable portion of HMX increased by 43 percent in the single treatment used in the limit test with weathered/aged HMX amended soil.

2,4-DNT acetonitrile-extractable concentrations in freshly amended soils averaged 82 (range: 48-86) percent of nominal concentrations (Table 5). 2,4-DNT ATCLP-extractable concentrations averaged 43 (range: 19-84) percent of acetonitrile-extractable concentrations (Table 5). 2,4-DNT ATCLP-extractable concentrations averaged 55 (range: 46-70) percent of acetonitrile-extractable concentrations (Table 6).

Weathering/aging of amended soil decreased acetonitrile-extractable 2,4-DNT concentrations, on average, by 46, and ATCLP-extractable 2,4-DNT concentrations by 18 percent compared with respective concentrations in freshly amended soil.

2,6-DNT acetonitrile-extractable concentrations in freshly amended soils averaged 126 (range: 80-267) percent of nominal concentrations (Table 7). 2,6-DNT ATCLP-extractable concentrations increased proportionally with their acetonitrile-extractable concentrations and averaged 48 (range: 27-65) percent of acetonitrile-extractable concentrations (Table 7). 2,6-DNT ATCLP-extractable concentrations averaged 49 (range: 40-62) percent of acetonitrile-extractable concentrations in weathered/aged amended soil (Table 8).

Weathering/aging of amended soil decreased acetonitrile-extractable 2,6-DNT concentrations, on average, by 76% compared with acetonitrile-extractable concentrations in freshly amended soil, while ATCLP-extractable 2,6-DNT concentrations decreased, on average, by 81 percent compared with freshly amended soil.

TNB recovery was greatly reduced in treatments below 45 mg kg^{-1}. TNB acetonitrile-extractable concentrations in freshly amended soil averaged 62 (range: 25-100) percent of nominal concentrations (Table 9). TNB ATCLP-extractable concentrations averaged 73 (range: 56-86) percent of acetonitrile-extractable concentrations (Table 9). These values do not include data for 8 mg kg^{-1} nominal treatment concentration, which had TNB recovery in one (0.13 mg kg^{-1}) out of three replicates producing an average ATCLP-extractable value of 0.043 mg kg^{-1} (Table 9). TNB ATCLP-extractable concentrations averaged 55 (range: 19-93) percent of acetonitrile-extractable concentrations in weathered/aged amended soil (Table 10).

Weathering/aging of amended soil decreased acetonitrile-extractable TNB concentrations, on average, by 54% compared with acetonitrile-extractable concentrations in freshly amended soil, while ATCLP extractable TNB concentrations decreased, on average, by 59 percent compared with freshly amended soil.

3.2 Range-Finding Toxicity Tests.

Either RDX or HMX had little or no effect on adult survival in the range-finding tests in all treatment concentrations. Juvenile numbers were reduced by 21 ($p = 0.118$) percent in 1,000 mg kg^{-1} RDX treatment and by 30 ($p < 0.041$) percent in both 5,000 and 10,000 mg kg^{-1} RDX treatments compared to control. There were no adverse effects on juvenile production in any of the HMX treatments. Results of range finding test showed that 2,4-DNT significantly ($p < 0.0001$) reduced adult survival at 100 mg kg^{-1}. No adults survived at the higher concentrations. Juvenile numbers were reduced by 19 ($p = 0.03$) and 81 ($p < 0.0001$) percent in 10 and 50 mg kg^{-1} treatments, respectively compared to control. No juveniles were produced at the higher concentrations. Range-finding tests with 2,6-DNT showed that adult survival was reduced at 50 mg kg^{-1}. No adults survived at the higher concentrations. Juvenile numbers were reduced by 25 ($p = 0.001$) and 72 ($p < 0.0001$) percent in 10 and 50 mg kg^{-1} treatments, respectively compared to control. No juveniles were produced at the higher concentrations.

Adult survival in the range-finding test with TNB was significantly reduced at 100 mg kg^{-1} ($p = 0.048$). Juvenile numbers were reduced by 19 ($p = 0.153$) and 75 ($p < 0.0001$) percent in 50 and 100 mg kg^{-1} treatments, respectively compared to control. Juvenile numbers were reduced by approximately 99 percent in 500 and 1,000 mg kg^{-1} treatments. No juveniles were produced at the higher concentrations. Results of these range-finding tests allowed us to determine treatment concentrations for the definitive test shown in Tables 2-10.

3.3 Definitive Toxicity Tests.

Definitive studies using the Enchytraeid Reproduction Tests (ERT) were conducted to assess the effects of RDX, HMX, 2,4-DNT, 2,6-DNT, or TNB on the reproduction of the enchytraeid worm *E. crypticus*. Adult *E. crypticus* were exposed in SSL soil to a range of concentrations for each EM, in independent investigations. Measurement endpoints were assessed using 6-9 treatment concentrations determined from the range-finding studies and included number of surviving adults after 14 days and number of juveniles after 28 days. All ecotoxicological parameters were estimated using measured chemical concentrations for each treatment level.

Test results complied with the validity criteria defined in the ISO test guideline. Mean adult survival in negative controls was 98% in 2,6-DNT freshly amended soil and 100% in all other tests. The mean juvenile production in negative controls ranged from 809 to 1500 juveniles, and the coefficient of variation ranged from 5.4 to 16.2 percent. Direct comparisons of the results of positive control are not possible because ERT is a new test and no reference values for natural soils are available from the literature. Juvenile production in positive controls ranged from 56 to 67 percent reduction from negative controls and was within the baseline established for the laboratory culture of *E. crypticus*. These results confirmed that the toxicological effects determined in the definitive tests were most likely due to test EM treatments. All reported ecotoxicological parameters have been calculated based on measured concentrations.

3.3.1 Toxicity of RDX.

Results of RDX toxicity testing in freshly amended and weathered/aged amended SSL soil are shown in Table 11. Adult *E. crypticus* survival was not affected in any RDX treatment concentrations producing the unbounded NOEC values for RDX in freshly amended soils of 21,383 mg kg^{-1} based on acetonitrile-extractable concentrations (Table 12) and 107 mg kg^{-1} based on ATCLP extractable concentrations. The acetonitrile-extractable concentration based unbounded NOEC value for RDX in weathered/aged amended soil was 18,347 mg kg^{-1} (Table 12). The unbounded NOEC value for RDX in weathered/aged amended soil based on ATCLP extractable concentrations was 89 mg kg^{-1}.

Juvenile production bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were, respectively 1,194 and 2,203 mg kg^{-1} in freshly amended soil, and 2,379 and 3,985 mg kg^{-1} in weathered/aged amended soil (Table 12). The ATCLP based

NOEC and LOEC values for juvenile production were almost identical in both freshly amended and weathered/aged amended soils because these concentrations exceeded RDX solubility in water. These values were, respectively 114.8 and 114.7, and 87 and 86 mg kg^{-1}.

Table 11. Adult survival and juvenile production (mean, n = 4) in freshly amended and weathered/aged RDX amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.

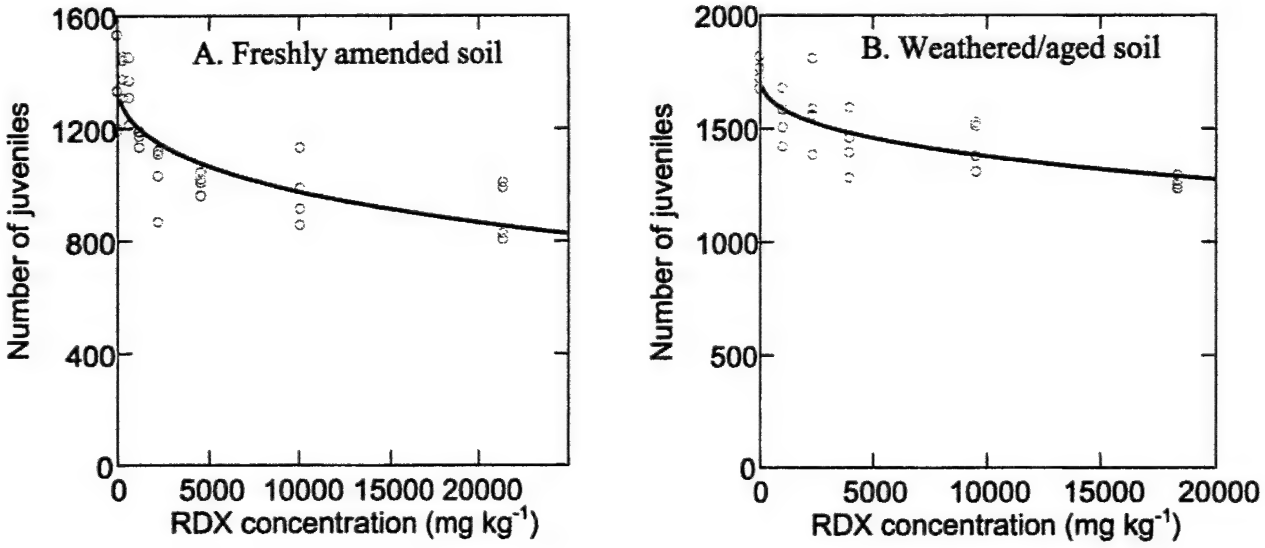
Concentration in freshly amended soil (mg kg^{-1})	Number of Adults	Number of Juveniles	Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Number of Adults	Number of Juveniles	Standard Error
Negative control	10	1278.5	35	Negative control	10	1120.5	84
Acetone control	10	1313.8	80	Acetone control	10	1748.5	30
Positive control	10	419.8	24	Positive control	10	488.8	33
304	9.8	1395.3	34	1048	10	1549.8	55
656	10	1336.5	50	2379	9.8	1587.3	87
1194	10	1170.5	13	3985	10	1434.0	65
2203	10	1032.5	58	9549	10	1433.8	53
4558	10	1009.0	17	18347	9.8	1264.5	13
10062	10	973.8	59				
21383	10	908.8	53				

Table 12. Ecotoxicological parameters (mg kg^{-1}) for RDX determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.

Exposure	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh	21,383	>21,383	1,194	2,203	3,715	51,413
p or 95% C.I.			0.055	0.001	0-8,100	6,336-96,491
R^2					0.990	0.990
Weathered/aged	18,347	>18,347	2,379	3,985	8,797	142,356
p or 95% C.I.			0.056	0.001	761-16,834	0-373,753
R^2					0.995	0.995

Concentration-response relationships for juvenile production in freshly amended and weathered/aged RDX amended soils determined by nonlinear regressions are shown in Figure 1. Logistic (Gompertz) model had the best fit for the data in tests with both freshly amended (Figure 1A) and weathered/aged amended (Figure 1B) soils. Overall, reproduction was higher in weathered/aged RDX amended soils (Table 11). Juvenile production EC_{20} values were 3,715 and 8,797 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The difference between these values was not statistically significant based on 95% confidence intervals (Table 12). Juvenile production EC_{50} values were 51,413 and 142,356 mg kg^{-1} in freshly amended and weathered/aged soils, respectively. The highest RDX concentration of 21,383 mg kg^{-1} used in the test with freshly amended soil, and 18,347 mg kg^{-1} used in the test with weathered/aged amended soil resulted only in 31 and 28 percent reductions in the number of juveniles produced, respectively compared to carrier control. For that reason, nonlinear regression model estimated large range for 95% C.I. for both EC_{50} parameters (Table 12) indicating high uncertainty in these point estimates.

Figure 1. Effects of RDX on juvenile production in freshly amended (A) and weathered/aged (B) RDX amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.



All treatment concentrations used for toxicity assessments with *E. crypticus* in SSL soil were above the RDX solubility level in water (42.3 mg L^{-1} at 20°C; Roberts and Hartley, 1992) producing uniformed ATCLP concentrations across the range. This precluded determinations of the concentration-response relationship on the basis of water extractable (ATCLP) RDX portion in both freshly amended and weathered/aged amended SSL soil.

3.3.2 Toxicity of HMX.

Results of HMX toxicity testing in freshly amended and weathered/aged amended SSL soils are shown in Table 13. Adult *E. crypticus* survival was not affected in any HMX concentrations tested. Juvenile *E. crypticus* production was stimulated at higher HMX concentrations in freshly amended soil (Figure 2). The increase was statistically significant ($p < 0.05$) at 2,211 mg kg^{-1} and higher concentrations producing a bounded NOEC ($p = 0.109$) of 1,491 mg kg^{-1} and unbounded NOAEC (No Observed Adverse Concentration) of 21,750 mg kg^{-1} (Table 14). Results of the limit test showed that exposure of *E. crypticus* in weathered/aged HMX amended soil did not affect reproduction producing an unbounded NOEC ($p = 0.186$) of 17,498 mg kg^{-1}. Similar to RDX, all HMX treatment concentrations used for toxicity assessments were above the HMX solubility level in water (6.63 mg L^{-1} at 20°C; Roberts and Hartley, 1992).

Table 13. Adult survival and juvenile production in freshly amended and weathered/aged HMX amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.

Concentration in freshly amended soil (mg kg^{-1})	Mean* Adults	Mean Juveniles	Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error
Negative control	10	808.8	31.8	Negative control	10	1116.3	33
Acetone control	10	737.3	45.4	Acetone control	10	1468.1	69
Positive control	10	292.0	19.4	Positive control	10	488.8	33
348	9.8	741.5	29.2	17498	10	1359.4	33
642	10	825.5	15.9				
1491	10	847.8	19.5				
2211	9.8	922.8	96.5				
5785	10	1151.0	19.9				
10586	10	986.8	33.9				
21750	10	1143.0	56.3				

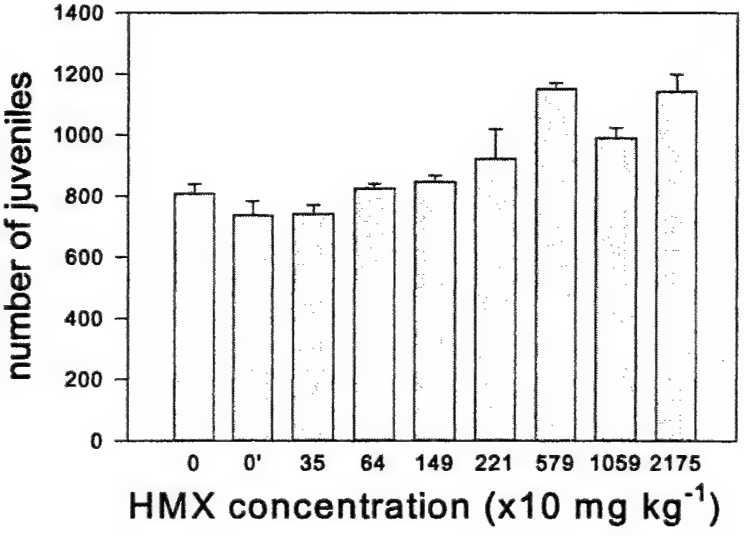
Table notes:
*Means are based on n = 4 for all treatments in freshly amended soils. Means in the limit test using weathered/aged HMX amended soil are based on n = 8 for carrier control and one treatment concentration of 17498 mg kg^{-1}; and n = 4 for the negative and positive controls.

Table 14. Ecotoxicological parameters (mg kg^{-1}) for HMX determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.

Exposure	Adult survival		Juvenile production			
	NOEC	LOEC	NOAEC	LOAEC	EC_{20}	EC_{50}
Fresh	21,750	>21,750	21,750	>21,750	ND	ND
Weathered/aged	17,498	>17,498	17,498	>17,498	LT	LT

Table notes:
ND, Not Determined. ECp values could not be determined due to stimulation of juvenile production in all treatment concentrations.
LT, Limit Test is based on data comparison between carrier control and one treatment concentration of 17,498 mg kg^{-1}.

Figure 2. Effect of HMX (mean and S.E., n = 4) on juvenile production by *Enchytraeus crypticus* in freshly amended Sassafras sandy loam soil. Controls shown are negative (0) and carrier (0'). All concentrations are based on acetonitrile extraction using USEPA Method 8330.



3.3.3 Toxicity of 2,4-DNT.

Adult *E. crypticus* survival and juvenile production were affected in 2,4-DNT amended SSL soil within the concentrations ranges selected from the results of range-finding test (Table 15). For adult survival in freshly amended soil, the bounded NOEC and LOEC values for 2,4-DNT based on acetonitrile-extractable concentrations were 40.9 and 55.0 mg kg^{-1}, respectively.

The bounded NOEC and LOEC values based on water extractable (ATCLP) concentrations were 8.1 and 33.4 mg kg^{-1}, respectively. For adult survival in weathered/aged amended soil, the bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were 37 and 72 mg kg^{-1}, respectively. No adults survived in the 179 mg kg^{-1} treatment. The bounded NOEC and LOEC values based on ATCLP extraction were 20.5 and 46.1 mg kg^{-1}, respectively (Table 16).

Table 15. Adult survival and juvenile production (mean, n = 4) in freshly amended and weathered/aged 2,4-DNT amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.

Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error
Negative control	10	823.5	39	Negative control	10	1500.0	79
Acetone control	9.8	1076.5	61	Acetone control	10	1449.0	122
Positive control	9.3	351.5	23	Positive control	10	578.3	40
0.95	10	1159.0	79	3.0	10	1447.5	72
3.0	10	1116.0	73	5.2	10	1478.0	67
6.5	10	984.0	52	11.5	10	1116.5	24
9.9	10	971.3	71	21.5	10	1093.5	79
20.3	9.8	870.8	109	31.0	10	650.3	82
40.9	10	489.5	45	37.3	9.8	395.0	58
55.0	8.3	218.8	49	71.7	8.3	45.0	12
64.7	5.8	115.0	14	178.7	0.0	0.0	

Juvenile production bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were, respectively 9.9 and 20.3 mg kg^{-1} in freshly amended soils, and 5.2 and 11.8 mg kg^{-1} in weathered/aged amended soils. Juvenile production bounded NOEC and LOEC values based on ATCLP extractable concentrations were, respectively 4.96 and 8.13 mg kg^{-1} in freshly amended soils, and 2.42 and 5.2 mg kg^{-1} in weathered/aged amended soils (Table 16).

Table 16. Ecotoxicological parameters (mg kg^{-1}) for 2,4-DNT determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

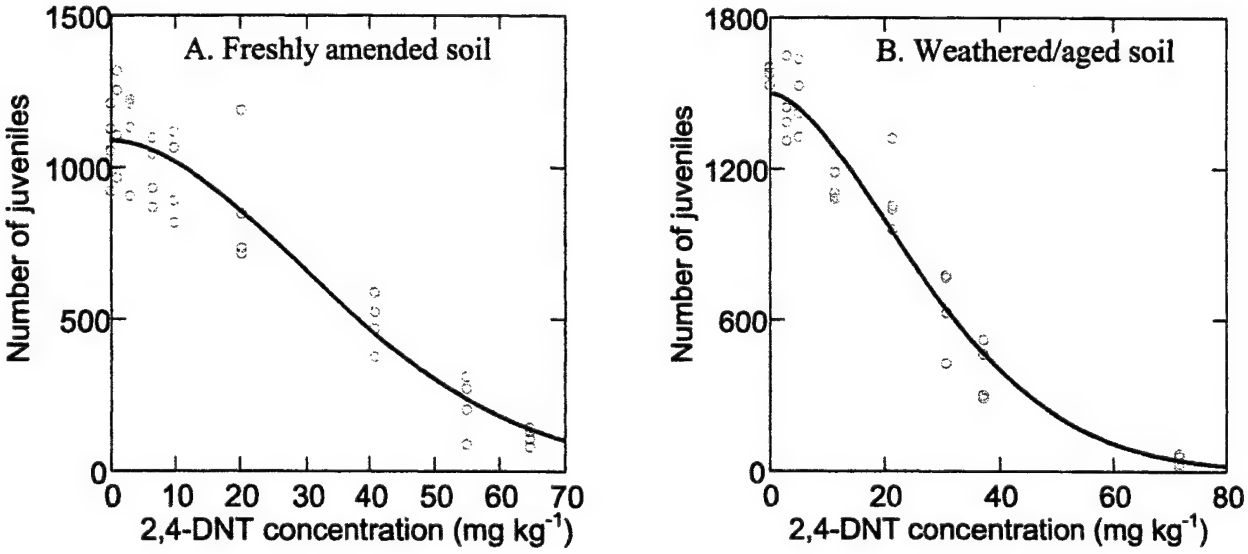
Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	40.9	55.0	9.9	20.3	19	36
p or 95% C.I.	0.659	0.013	0.271	0.037	13 - 26	30 - 41
R^2					0.980	0.980
ATCLP extraction	8.1	33.4	4.96	8.13	3	11
p or 95% C.I.	0.659	0.013	0.271	<0.0001	1 - 5	7 - 14
R^2					0.971	0.971
Weathered/aged						
Acetonitrile extraction	37	72	5.2	11.8	14	27
p or 95% C.I.	0.711	0.015	0.318	<0.0001	10 - 18	24 - 31
R^2					0.985	0.985
ATCLP extraction	20.5	46.1	2.42	5.2	7	14
p or 95% C.I.	0.711	0.015	0.318	<0.0001	4 - 9	12 - 16
R^2					0.983	0.983

Concentration-response relationships for juvenile production in freshly amended and weathered/aged 2,4-DNT amended soils determined by nonlinear regressions are shown in Figure 3. Logistic (Gompertz) model had the best fit for the data in tests with both freshly amended (Figure 3A) and weathered/aged amended (Figure 3B) soils. Overall, reproduction was higher in weathered/aged 2,4-DNT amended soils. Juvenile production EC_{20} values based on acetonitrile-extractable concentrations were 19, and 14 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively. Juvenile production EC_{50} values based on acetonitrile-extractable concentrations were 36 and 27 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 16). Juvenile production EC_{20} values based on ATCLP extractable concentrations were 3, and 7 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 16). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 11 and 14 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 16). The differences among these values were not statistically significant based on 95% confidence intervals (Table 16) indicating that the 3-month weathering and aging of 2,4-DNT amended soils did not affect the toxicity of this EM to *E. crypticus*.

Coefficients of determinations (R^2) for acetonitrile-extractable and ATCLP based extractions determined in nonlinear regression analyses of the reproduction toxicity data (EC_{20} levels) from studies with freshly amended and weathered/aged 2,4-DNT amended soils were

compared to determine which chemical measure of exposure better correlates with toxicity. The values of coefficients in freshly amended soils were 0.980 and 0.971 for acetonitrile-extractable and ATCLP based extractions, respectively. These values in weathered/aged 2,4-DNT amended soils were 0.985 and 0.983 for acetonitrile-extractable and ATCLP based extractions, respectively. These comparisons show that coefficients were very similar in both exposure types indicating that neither extraction method had an advantage in characterizing 2,4-DNT bioavailability to *E. crypticus*.

Figure 3. Effects of 2,4-DNT on juvenile production in freshly amended (A) and weathered/aged (B) 2,4-DNT amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.



3.3.4 Toxicity of 2,6-DNT.

Results of toxicity testing in 2,6-DNT freshly amended and weathered/aged amended SSL soils are shown in Table 17. Adult *E. crypticus* survival was not affected in any 2,6-DNT concentrations in freshly amended SSL soil producing the unbounded NOEC value of 64 mg kg^{-1} based on acetonitrile-extractable concentration, and 40.5 mg kg^{-1} based on ATCLP extractable concentration. For adult survival in weathered/aged amended soil, the bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were 37.4 and 108.3 mg kg^{-1}, respectively. The bounded NOEC and LOEC values based on ATCLP extraction were 17.4 and 66.9 mg kg^{-1}, respectively (Table 18).

Table 17. Adult survival and juvenile production (mean, n = 4) in freshly amended and weathered/aged 2,6-DNT amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.

Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error
Negative control	9.8	882.8	35	Negative control	10	983.3	45
Acetone control	9.8	893.0	61	Acetone control	9.8	1145.0	13
Positive control	9.8	356.5	25	Positive control	9.5	411.3	18
5.3	10	830.5	39	3.7	9.8	1161.8	90
7.7	10	966.8	119	9.5	9.5	1111.8	82
9.4	10	859.3	45	13.9	9.3	969.5	71
12.9	10	866.0	32	18.1	9.8	947.5	37
20.0	10	753.8	35	37.4	9.8	346.8	100
40.2	10	673.8	54	108.3	1.7	3.0	2.7
51.1	10	560.0	85				
64.0	9.5	306.5	22				

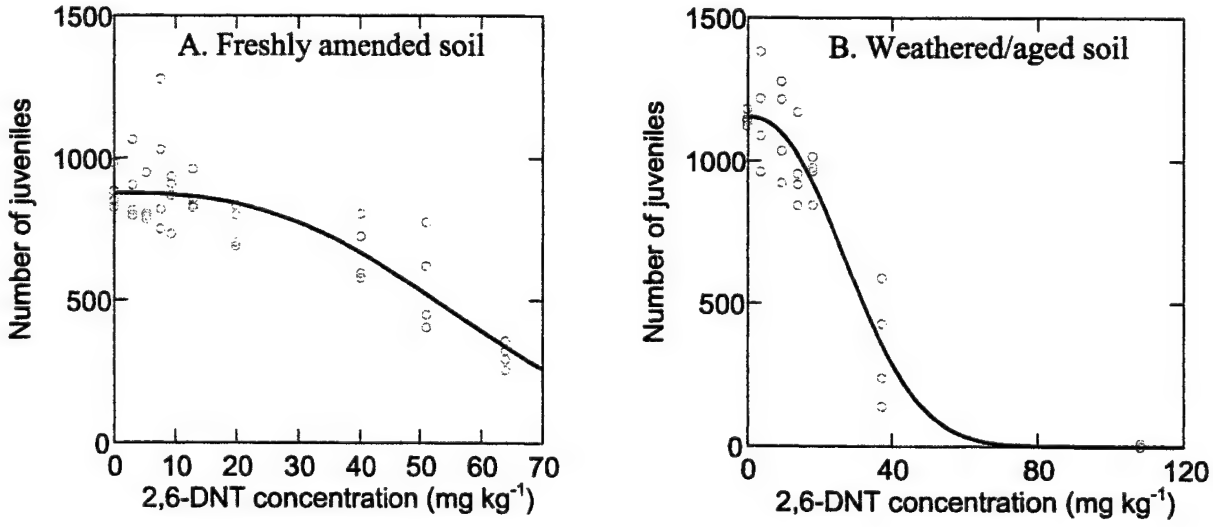
Table 18. Ecotoxicological parameters (mg kg^{-1}) for 2,6-DNT determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	64	>64	20.0	40.2	37	57
p or 95% C.I.	0.369		0.136	0.019	28 - 47	51 - 63
R^2					0.980	0.980
ATCLP extraction	40.5	>40.5	10.6	24.8	24	36
p or 95% C.I.	0.292		0.071	0.005	17 - 30	32 - 40
R^2					0.979	0.979
Weathered/aged						
Acetonitrile extraction	37.4	108.3	18.1	37.4	18	29
p or 95% C.I.	1.000	<0.0001	0.055	<0.0001	13 - 23	25 - 34
R^2					0.984	0.984
ATCLP extraction	17.4	66.9	9.6	17.4	9	14
p or 95% C.I.	1.000	<0.0001	0.055	<0.0001	7 - 12	13 - 16
R^2					0.983	0.983

Juvenile production bounded NOEC and LOEC values based on acetonitrile-extractable concentrations were, respectively 20.0 and 40.2 mg kg^{-1} in freshly amended soils, and 18.1 and 37.4 mg kg^{-1} in weathered/aged soils. Juvenile production bounded NOEC and LOEC values based on ATCLP extractable concentrations were, respectively 10.6 and 24.8 mg kg^{-1} in freshly amended soils, and 9.6 and 17.4 mg kg^{-1} in weathered/aged soils (Table 18).

Logistic (Gompertz) model had the best fit for the data in tests with both freshly amended (Figure 4 A) and weathered/aged amended (Figure 4 B) soils. Juvenile production EC_{20} values based on acetonitrile-extractable concentrations were 37, and 18 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively. Juvenile production EC_{50} values based on acetonitrile-extractable concentrations were 57 and 29 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 18). Juvenile production EC_{20} values based on ATCLP extractable concentrations were 24, and 9 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 18). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 36 and 14 mg kg^{-1} in freshly amended and weathered/aged amended soils, respectively (Table 18). The differences among these values were statistically significant based on 95% confidence intervals (Table 18) indicating that the 3-month weathering/aging of 2,6-DNT amended soils increased the toxicity of this energetic material to *E. crypticus*.

Figure 4. Effects of 2,6-DNT on juvenile production in freshly amended (A) and weathered/aged (B) 2,6-DNT amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.



Coefficients of determinations (R^2) for acetonitrile-extractable and ATCLP based extractions determined in nonlinear regression analyses of the reproduction toxicity data (EC_{20} levels) from studies with freshly amended and weathered/aged 2,6-DNT amended soils were compared to determine which chemical measure of exposure better correlated with toxicity. The values of coefficients in freshly amended soils were 0.980 and 0.979 for acetonitrile-extractable and ATCLP based extractions, respectively (Table 18). These values in weathered/aged 2,6-DNT amended soils were 0.984 and 0.983 for acetonitrile-extractable and ATCLP based extractions, respectively (Table 18). These comparisons show that coefficients were very similar in both exposure types indicating that neither extraction method had an advantage in characterizing 2,6-DNT bioavailability to *E. crypticus*.

3.3.5 Toxicity of TNB.

TNB affected both adult *E. crypticus* survival and juvenile production in amended SSL soil within the concentrations ranges selected from the results of range-finding test (Table 19). Adult survival in freshly amended soil was not affected up to 45 mg kg^{-1} (bounded NOEC) acetonitrile extractable treatment concentration. No adults survived after a 14-day exposure to TNB in 107 mg kg^{-1} (bounded LOEC) acetonitrile extractable treatment concentration. The bounded NOEC and LOEC values based on water extractable (ATCLP) concentrations were 30.2 and 83.7 mg kg^{-1}, respectively (Table 20). Weathering/aging of TNB amended soil reduced the toxicity of TNB to *E. crypticus* adults. The bounded NOEC and LOEC values in weathered/aged soils based on acetonitrile-extractable concentrations were 75.8 and 176 mg kg^{-1}, respectively. The bounded NOEC and LOEC values based on ATCLP concentrations were 55.8 and 143.4 mg kg^{-1}, respectively (Table 20).

Table 19. Adult survival and juvenile production (mean, n = 4) in freshly amended and weathered/aged TNB amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.

Concentration in freshly amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error	Concentration in weathered/aged amended soil (mg kg^{-1})	Mean Adults	Mean Juveniles	Standard Error
Negative control	10	824.8	61	Negative control	10	1387.3	61
Acetone control	10	804.3	35	Acetone control	10	1465.3	36
Positive control	9.3	329.3	31	Positive control	9.0	579.0	78
2.3	10	780.3	19	0.6	9.8	1355.0	81
2.6	10	958.0	56	1.3	9.5	1501.8	24
3.9	10	654.3	24	8.8	9.3	1166.3	128
13.6	9.8	374.3	35	75.8	9.8	61.5	18
45.0	10	238.0	62	176.3	0	0	
107.0	0	0		304.7	0	0	
221.0	0	0					
385	0	0					

Table 20. Ecotoxicological parameters (mg kg^{-1}) for TNB determined in freshly amended and weathered/aged amended Sassafras sandy loam soil using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using U.S. EPA Method 8330 and water extraction using Adapted Toxicity Characteristic Leaching Procedure (ATCLP).

Exposure assessment	Adult survival		Juvenile production			
	NOEC	LOEC	NOEC	LOEC	EC_{20}	EC_{50}
Fresh						
Acetonitrile extraction	45.03	107	2.6*	3.9*	5	11
p or 95% C.I.	1.000	<0.0001	0.010	0.012	3 - 7	7 - 16
R^2					0.975	0.975
ATCLP extraction	30.2	83.7	<2.4	2.4**	1.3	9
p or 95% C.I.	1.000	<0.0001		0.001	0.1 - 2.5	5 - 12
R^2					0.980	0.980
Weathered/aged						
Acetonitrile extraction	75.8	176	1.3	8.8	9	22
p or 95% C.I.	1.000	<0.0001	0.722	0.009	4 - 14	13 – 32
R^2					0.988	0.988
ATCLP extraction	55.8	143.4	0.24	3.35	3	11
p or 95% C.I.	1.000	<0.0001	0.722	0.009	1 – 6	5 – 17
R^2					0.988	0.988

Table notes:
*Values are No Observed Adverse Effect Concentration, NOAEC and Lowest Observed Adverse Effect Concentration, LOAEC due to a significant ($p = 0.01$) increase in juvenile production in 2.6 mg kg^{-1} treatment.
**Unbounded LOEC value. TNB concentrations using ATCLP extraction were below the method detection limit (MDL) in the preceding amended treatments 2.3 and 2.6 mg kg^{-1} based on acetonitrile extraction from freshly amended soils.

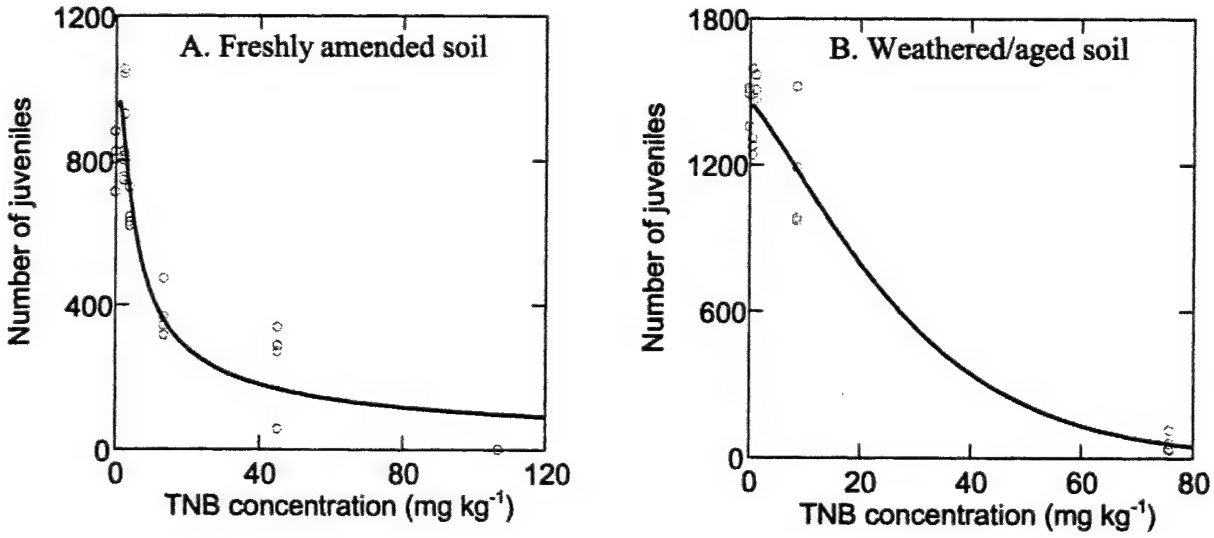
Juvenile production was stimulated at the lower treatment concentration of 2.6 mg kg^{-1} resulting in the 19 percent increase in the average number on juveniles compared with carrier control (Table 19). The increase was statistically significant ($p = 0.01$) producing a bounded NOEC value of 2.3 mg kg^{-1} and a bounded LOEC value of 2.6 mg kg^{-1} based on acetonitrile-extractable concentration. Statistically significant ($p = 0.012$) reduction in number of juveniles compared with carrier control occurred in 3.9 mg kg^{-1} treatment (Table 19), which produced a bounded NOAEC value of 2.6 mg kg^{-1} and bounded LOAEC value of 3.9 mg kg^{-1} based on acetonitrile-extractable concentration (Table 20).

TNB concentrations using ATCLP extraction were below the method detection limit (MDL) in the first two amended treatments (2.3 and 2.6 mg kg^{-1} based on acetonitrile extraction) in freshly amended soils. The first treatment with positively detected TNB concentrations using ATCLP extraction had a significantly ($p = 0.001$) decreased juvenile

production compared with carrier control producing an unbounded LOEC of 2.4 mg kg^{-1} for freshly amended soils. Juvenile production bounded NOEC and LOEC values in weathered/aged soils were 1.3 and 8.8 mg kg^{-1}, respectively based on acetonitrile extraction, and 0.24 and 3.35 mg kg^{-1}, respectively based on ATCLP extraction (Table 20).

The logistic model with hormetic parameter (hormetic model) had the best fit for the data from toxicity tests with TNB freshly amended SSL soil due to stimulation of juvenile production at the lower treatment concentration of 2.6 mg kg^{-1} (Figure 5 A). Juvenile production EC_{20} and EC_{50} values based on acetonitrile-extractable concentrations were 5, and 11 mg kg^{-1}, respectively (Table 20). Juvenile production EC_{20} and EC_{50} values based on ATCLP extractable concentrations were 1.3, and 9 mg kg^{-1}, respectively (Table 20). The logistic (Gompertz) model had the best fit for data in tests with weathered/aged TNB amended soils (Figure 5 B). Overall, reproduction was higher in weathered/aged amended soils. Juvenile production EC_{20} and EC_{50} values based on acetonitrile-extractable concentrations were 9 and 22 mg kg^{-1}, respectively (Table 20). Juvenile production EC_{20} and EC_{50} values based on ATCLP extractable concentrations were 3 and 11 mg kg^{-1}, respectively (Table 20). The differences between ECp values for freshly amended and weathered/aged TNB amended soils were not statistically significant based on 95% confidence intervals (Table 20) indicating that the 3-month weathering/aging of TNB amended soils did not affect the toxicity of this energetic material to *E. crypticus*.

Figure 5. Effects of TNB on juvenile production in freshly amended (A) and weathered/aged (B) TNB amended Sassafras sandy loam soils determined in toxicity testing using Enchytraeid Reproduction Test with *Enchytraeus crypticus*. Concentrations are based on acetonitrile extraction using USEPA Method 8330.



Coefficients of determinations (R^2) for acetonitrile-extractable and ATCLP based extractions determined in nonlinear regression analyses of the reproduction toxicity data (EC_{20} levels) from studies with freshly amended and weathered/aged TNB amended SSL soils were compared to determine which chemical measure of exposure better correlates with toxicity. The values of coefficients in freshly amended soils were 0.975 and 0.980 for acetonitrile-extractable and ATCLP based extractions, respectively. These values in weathered/aged TNB amended soils were 0.988 for both acetonitrile-extractable and ATCLP based extractions (Table 20). These comparisons show that coefficients were very similar in both exposure types indicating that neither extraction method had an advantage in characterizing TNB bioavailability to *E. crypticus*.

4. DISCUSSION

Development of ecotoxicological benchmarks for energetic soil contaminants has become a critical need in recent years. These benchmarks are required for derivation of ecological soil screening levels (Eco-SSLs) for use in Ecological Risk Assessment (ERA) of contaminated sites (USEPA, 2000). Eco-SSLs represent concentrations of chemicals in soil that, when not exceeded, will be theoretically protective of terrestrial ecosystems within specific soil boundary conditions from unacceptable harmful effects. An extensive review of literature determined that there was insufficient information for energetic material contaminants in soil to generate Eco-SSL benchmarks for soil invertebrates (USEPA, 2000). The majority of soil toxicity tests that were reported in literature utilized standard artificial soil with high organic matter content (10%). In contrast, our toxicity studies designed to specifically fill this knowledge gap, used a natural soil that meet the criteria for Eco-SSL development, in large part because it has characteristics supporting relatively high bioavailability of EMs. In addition, the weathering and aging procedure applied to soils loaded with the range of EM concentrations allowed us to more realistically assess the toxicity under conditions more closely resembling the potential toxic effects of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in the field.

4.1 Chemical Analysis of Energetic Materials in Soil.

Derivation of Eco-SSL values prioritizes ecotoxicological benchmarks that are based on measured soil concentration of a chemical over those based on nominal concentrations (USEPA, 2000). In this study, the exposure concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in soil were analytically determined in all definitive toxicity tests. Chemical analysis utilized the USEPA Method 8330 based on acetonitrile extraction of EMs from soil and measured acetonitrile-extractable chemical concentration. Results from acetonitrile extraction of freshly amended soils showed good correlation between nominal and measured concentrations for the five energetic materials, confirming that the soil amendment procedure used in toxicity tests was appropriate and that the USEPA Method 8330 was efficient for quantifying the amount of energetic materials in soil.

An additional procedure that measures the water extractable portion of each EM in amended soil was performed using the Adapted Toxicity Characteristic Leaching Procedure (ATCLP). This water extractable portion of each EM was perceived to measure the bioavailable

fraction of chemicals in soil pore water that is potentially better correlated with toxicity as compared to acetonitrile extracted chemical measure. ATCLP extractable concentrations of 2,6-DNT, and TNB freshly amended in SSL soil increased proportionally with their respective acetonitrile-extractable concentrations. In contrast, RDX and HMX ATCLP extractable concentrations decreased proportionally with their respective acetonitrile-extractable concentrations with less than one percent recovery in soils freshly amended at or above 10,000 mg kg^{-1} RDX, and at or above 1,200 mg kg^{-1} HMX. These low ATCLP-based recoveries reflected the low water solubility of both compounds, which were reported for RDX at 42 mg L^{-1} at 20°C (Sikka *et al.*, 1980) and at 60 mg L^{-1} at 25°C (Banerjee *et al.*, 1980). The water solubility of HMX was reported between 5 and 6.6 mg L^{-1} at 25°C and 20°C, respectively (Glover and Hoffsommer, 1973; McLellan *et al.*, 1992).

Assessment of the EM toxicity to *E. crypticus* for Eco-SSL development included studies with weathered and aged EM amended soils to simulate more closely the exposure effects in the field. Weathering/aging of chemicals in soil may reduce exposure of soil invertebrates to EMs due to photodecomposition, hydrolysis, reaction with organic matter, sorption, precipitation, immobilization, occlusion, microbial transformation and other fate processes that commonly occur at contaminated sites. These fate processes can reduce the amount of chemical that is bioavailable, compared to tests conducted with freshly amended soils, or may reveal increased toxicity due to the presence of more toxic transformation products.

Weathering/aging of amended soils decreased acetonitrile-extractable RDX or HMX concentrations after the 3-month weathering/aging period, on average, by 7 or 20 percent, respectively compared with acetonitrile-extractable concentrations in freshly amended soils. These findings are in agreement with the study results of Jones *et al.* (1995) who reported a limited, 10 percent mineralization of RDX in contaminated soil augmented with *Rhodococcus* bacterial strain. Weathering/aging of RDX amended soils decreased average water extractable fraction by 16%. In contrast with RDX, weathering/aging of HMX amended soils increased water extractable fraction of HMX by 43 percent in the single treatment used in the limit test with weathered/aged HMX amended soil. Overall, water extractable fractions of RDX and HMX remained low after the 3-month weathering/aging period and were 3% and 0.1%, respectively of acetonitrile-extractable concentrations. These results confirm conclusions by Rosenblatt *et al.*, (1991), and Hawari and Halasz (2002), that RDX or HMX transformation is limited under aerobic conditions.

Weathering/aging of amended soils also decreased acetonitrile-extractable concentrations of 2,4-DNT, 2,6-DNT, or TNB. Concentration of 2,4-DNT decreased by approximately 50 percent during the three-months procedure and was independent of the initial acetonitrile-extractable concentrations used in this study. Weathered/aged amended SSL soil used in the phytotoxicity assessment portion of this investigation were analyzed for presence of metabolic products of nitroaromatic EMs transformation. These analyses identified two transformation metabolites of 2,4-DNT, including 2-amino-4-nitrotoluene (2-A-4 NT), and 4-amino-2-nitrotoluene (4-A-2 NT) in weathered/aged amended soil. Detection of these metabolites of 2,4-DNT confirmed that this EM was undergoing transformation. Bacteria able to mineralize 2,4-DNT, such as *Pseudomonas sp.* strain, have been isolated from a variety of

contaminated soils (Spain, 1995). Both 2,4-DNT and 2,6-DNT are readily biotransformed by *Pseudomonas sp.* and eventually eliminated as nitrite (Spanggord *et al.*, 1991; Kaplan, 1992; Haidor and Ramos, 1996). Transformation of 2,6-DNT during simulated weathering/aging procedure of amended soils was greater compared with 2,4-DNT transformation, and decreased 2,6-DNT concentrations by 76 percent. Transformation of TNB was inversely related to the initial acetonitrile-extractable concentration in amended soil. More than 80 percent decrease in TNB concentrations occurred in soils amended with concentrations below 100 mg kg^{-1}, while above that treatment level, TNB concentrations decreased by 20-30 percent. A metabolite of TNB, 3,5-dinitroaniline (3,5-DNA), was detected in weathered/aged amended SSL soil suggesting that TNB was undergoing microbial and/or photolytic transformation. Data analysis of ATCLP based extractions of nitroaromatic EMs in weathered/aged amended soils confirmed that the water extractable portions of TNB and DNTs in weathered/aged amended soils were significantly lower compared with freshly amended soils, presumably a result of fate processes in the amended soils undergoing weathering and aging. Overall, chemical analyses demonstrated that EM exposure conditions of *E. crypticus* in weathered/aged amended soils differed from those of freshly amended soils. The inclusion of weathering/aging component in the EM toxicity assessments allowed us to incorporate potential alterations in EM bioavailability at contaminated sites in the development of ecotoxicological benchmarks for soil invertebrates.

Coefficients of determinations (R^2) for acetonitrile and ATCLP based extractions determined in nonlinear regression analyses of the reproduction toxicity data from studies with freshly amended and weathered/aged amended soils were compared to determine which chemical measure of exposure better correlated with toxicity. These comparisons of coefficients of determinations showed that neither extraction method had an advantage for characterizing bioavailability of EMs to *E. crypticus*. This was true for both freshly amended and weathered/aged amended soils. This result supports our decision of developing draft Eco-SSLs for explosives contaminants in soil on the basis of acetonitrile extraction of test compounds. The acetonitrile extraction-based Eco-SSL values will be especially useful for Ecological Risk Assessment at contaminated sites because EM concentrations determined during site characterization are usually based on acetonitrile extraction by the USEPA Method 8330.

4.2 Toxicity of Energetic Materials to *E. crypticus* in Sassafras Sandy Loam Soil.

Definitive toxicity tests conducted with both freshly amended and weathered/aged amended soils showed that EM toxicity order based on EC_{20} values for juvenile production in tests with *E. crypticus* was TNB > 2,4-DNT > 2,6-DNT > RDX > HMX. Reproduction measurement endpoint in all tests was more sensitive compared with adult survival. This supported the Eco-SSL requirement of the use of reproduction endpoints for benchmark development (USEPA, 2000). Nitro-heterocyclic explosives RDX and HMX did not affect adult *E. crypticus* survival even at concentrations as high as 21,383 and 21,750 mg kg^{-1}, respectively. Juvenile production was affected by RDX but the toxicity was relatively low with EC_{20} and EC_{50} estimates of 3,715 and 51,413 mg kg^{-1} in freshly amended soils, respectively. Weathering and aging of RDX amended soil did not significantly affect its toxicity to *E. crypticus*. Exposure of *E. crypticus* to HMX in freshly amended SSL soil produced a significant stimulating effect on juvenile production (11-56% increase), which disappeared in weathered and aged soil. Stevens

et al. (2002) reported similar stimulating effect of HMX exposure on growth of the midge *Chironomus tentans*. Relatively low RDX toxicity and the absence of HMX toxicity to *E. crypticus* in SSL soil at concentrations tested in our study could be related to relatively low exposure concentrations of these EMs in pore water of amended soil, resulting from their low solubility levels in water. The solubility levels in water at 20°C of RDX and HMX are 42 and 6.6 mg L^{-1}, respectively (Sikka *et al.*, 1980; McLellan *et al.*, 1992). Additional research would be required to better understand the reasons for low toxicity of RDX to *E. crypticus* and elucidation of mechanisms of a stimulating response to HMX exposure.

Because this study was designed to produce benchmark data for development of Eco-SSLs for explosives contaminants in soil, the results of this study may not directly compare to those of other studies in the literature, since none of them were designed to specifically quantify EM toxicity to soil invertebrates under Eco-SSL conditions of testing. Literature on the toxicity of RDX to terrestrial organisms is scant, and discrepancies are often found regarding the toxicity of the same chemical to different organisms. Significant sublethal effects of RDX were observed on the reproduction of earthworm *Eisenia andrei* at concentrations as low as 95 mg kg^{-1} soil (Robidoux *et al.*, 2000). However, no effects were found on the mortality and reproduction of two terrestrial invertebrates enchytraeid worm *E. crypticus* and collembolan *Folsomia candida* in soils spiked with up to 1000 mg kg^{-1} RDX in soil (Schafer and Achazi, 1999). Furthermore, these studies were conducted either in standard artificial soil (Robidoux *et al.*, 2000), or in soil with relatively high (2.5-3.0% organic C) organic matter content (Schafer and Achazi, 1999), which limits their usefulness for describing natural systems or development of Eco-SSLs.

A hormetic response in freshly amended SSL soil was observed in our toxicity test with TNB amended soil. Similar hormetic responses were reported in explosives exposure studies for microbial nitrogen fixation activity at soil TNT concentrations of 200 and 400 mg kg^{-1} (Gong *et al.*, 1999), offspring production by *Daphnia magna* exposed to 0.08 mg L^{-1} TNT (Bailey *et al.*, 1985), egg production per female fathead minnow exposed to 6.3 mg L^{-1} RDX (Bentley *et al.*, 1977), and density of *Selanastrum capricornutum* cells, based on acetonitrile-extractable chlorophyll measures following HMX exposure ranging 36-572 mg L^{-1} (Bentley *et al.*, 1984). To date, no studies investigated the mechanisms responsible for stimulating effects of these explosives at specific concentrations. Stevens *et al.*, (2002) suggested that these mechanisms could include the direct effect on test organisms through the release of metabolic products of explosives that may have a specific effect on growth and reproduction, and indirect effects through increased supply of nitrogen for bacteria, fungi, or algae (an important food source for higher trophic levels) from mineralization of explosives.

Dinitrotoluenes (DNTs) and trinitrobenzene (TNB) are by-products of TNT production, which are present worldwide at munitions manufacturing and post-production sites. 2,4-DNT and 2,6-DNT are also aerobic metabolites of microbial degradation of TNT (Gorontzy, *et al.*, 1994; Spain *et al.*, 2000). Toxicity of nitroaromatic EMs tested to *E. crypticus* juvenile production was considerably greater (more than two orders of magnitude) compared with RDX and even greater compared with HMX. Juvenile production EC_{20} estimates ranged from 5 to 37 mg kg^{-1} in freshly amended soils, and from 9 to 20 mg kg^{-1} in weathered/aged amended soils. Comparison of our results to other studies is difficult because the toxicity of nitroaromatic

energetics, including 2,4-DNT, 2,6-DNT and TNB to soil invertebrates has not been sufficiently investigated. The majority of studies reported in the available literature focused primarily on the effects of TNT and/or its degradation products (Dodard *et al.*, 2003; Renoux *et al.*, 2000; Robidoux *et al.*, 2000; Sunahara, *et al.*, 2001; Rocheleau, *et al.*, 1999; Schafer and Achazi, 1999; Simini, *et al.*, 1995; Phillips, *et al.*, 1993). Dodard *et al.* (2003) in the study with *E. albidus* using OECD artificial soil determined EC_{50} value for TNT of 111 mg kg^{-1} for juvenile production. Phillips *et al.* (1993) reported 100 percent mortality in the earthworm *E. fetida* growth and survival test in USEPA standard artificial soil fortified with a mixture of EMs that included 30, 50, 62.5, and 20 mg kg^{-1} of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. Statistically significant ($p < 0.01$) sublethal effects (mass loss) were reported at concentrations 6, 10, 12.5, and 4 mg kg^{-1} of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. These results are in general agreement with findings of our investigations although direct comparisons of both studies are limited due to differences in the experimental designs.

Simini *et al.* (1995) assessed the toxicity of soil from Joliet Army Ammunition Plant contaminated with a mixture of EMs (which limits the direct comparisons with our study), including both nitroaromatic and nitro-heterocyclic compounds using earthworm *E. fetida* growth and survival test, among other bioassays. The highest soil concentrations measured at this site for TNB, 2,4-DNT and 2,6-DNT were 200, 117, and 8 mg kg^{-1}, respectively. Authors reported that TNT and TNB had greatest coefficients of determinations in all bioassays, including the earthworm test. Liner regression analyses R^2 values for TNB using earthworm test endpoints were 0.773 and 0.814 for the two locations investigated at the study site. These values for 2,4-DNT were 0.613 and 0.358, while 2,6-DNT had the weakest relationship to measurement points used with R^2 values of 0.082 and 0.293 for the two locations, respectively. Soil TNB and 2,4-DNT concentrations found at this site were within the range of concentrations tested in our study and the results are consistent with our findings. The weak relationship determined for 2,6-DNT is most likely due to very low concentrations of this EM measured at the investigated site.

Special consideration in assessing chemical toxicity for Eco-SSL development was given to the effects of weathering and aging of contaminant explosives in soil on exposure of soil invertebrates. Weathering and aging of amended soils significantly increased the toxicity of 2,6-DNT to *E. crypticus*, while toxicity of 2,4-DNT and TNB was unaffected. Dodard *et al.* (2003) reported a decrease in TNT toxicity to *E. albidus* on the LC_{50} basis for reproduction from 44 to 89 mg kg^{-1} in OECD artificial soil following a 21-day aging period. Specific mechanisms of changes in the toxicity of EMs in weathered/aged amended soil are unknown. Degradation products produced during the weathering and aging process may be more toxic to soil organisms compared with the parent material, and can be one of the factors contributing to the increased toxicity in weathered/aged amended soil. Dodard *et al.* (1999) investigated the toxicity of 2,4-DNT and 2,6-DNT, and their respective metabolites using the 15-min Microtox (*Vibrio fischeri*) and 96-h freshwater green alga (*S. capricornutum*) growth inhibition tests. The toxicities of DNTs were species-dependent: 2,4-DNT was more toxic than 2,6-DNT to *S. capricornutum* (comports with our results for *E. crypticus*), while the reverse was true in the test with *Vibrio fischeri*. The authors reported that the reduced metabolites of 2,6-DNT tested were less toxic compared to the toxicity of parent compound. However, certain partially reduced

metabolites of 2,4-DNT (4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene) were more toxic than the parent compound. Although these results cannot be directly compared to our study because the biotic reductive degradation pathway for 2,4-DNT and 2,6-DNT in aquatic environment contrasts with metabolic processes in the aerobic conditions of vadose zone simulated in our investigations, the reducing environment can exist in intermittently water-logged soil microsites, where more toxic metabolites of dinitrotoluenes transformation can be present. The higher toxicity of these metabolites may in part explain the increased toxicity of 2,6-DNT in weathered/aged amended SSL soil observed in our study. Overall results of our study showed that special consideration given to the effects of weathering and aging of energetic contaminants in soil for assessing toxicity was well justified. Benchmark values generated in this study will contribute to development of Eco-SSLs that better represent the exposure conditions of soil invertebrates at contaminated sites.

5. CONCLUSIONS

This study has produced ecotoxicological data for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB using ecologically relevant soil invertebrate species *E. crypticus*. Relative toxicity of the five EMs tested in this study was TNB > 2,4-DNT > 2,6-DNT > RDX > HMX. All ecotoxicological parameters were determined using measured chemical concentrations. This complies with USEPA preference for derivation of Eco-SSL values on the basis of measured soil concentration of a chemical over those based on nominal concentrations (USEPA, 2000). Chemical analyses of freshly amended soils using the USEPA Method 8330 showed good correlation between nominal and measured acetonitrile extracted concentrations for the five energetic materials confirming that the soil amendment procedure used in toxicity tests was appropriate and that this method was efficient for quantifying the amounts of energetic materials in soil. The water extractable portion of each EM, which was perceived to measure the immediately bioavailable fraction of chemicals in soil pore water, was determined using the Adapted Toxicity Characteristic Leaching Procedure (ATCLP). Comparisons of the results of nonlinear regression analyses of the toxicity tests data showed that neither extraction method had a statistical advantage for characterizing bioavailability and toxicity of EMs to *E. crypticus*. This result supports our decision to recommend developing Eco-SSLs for explosives contaminants in soil on the basis of acetonitrile extractable concentrations of test compounds.

A natural soil, Sassafras sandy loam was used in all toxicity tests. Sassafras sandy loam had low organic matter and clay contents, which fulfilled the USEPA requirement of using soil with characteristics that support relatively high contaminant bioavailability for developing conservative Eco-SSL values (USEPA, 2000). Weathering and aging of amended soils were incorporated into experimental design of toxicity testing to produce a soil microenvironment more similar to field conditions. Results of chemical analyses showed that exposure conditions of *E. crypticus* to EMs tested in weathered/aged amended soils differed from those of freshly amended soils due to significant transformation of TNB, 2,4-DNT, and 2,6-DNT, and the formation of transformation products, including 3,5-DNA, 2-A-4 NT, and 4-A-2 NT. The inclusion of weathering/aging component in the EM toxicity assessments allowed us to assess the potential alterations in EM bioavailability to *E. crypticus* at contaminated sites. In order to

provide a more complete information on ecotoxicological effects of energetic contaminants in soil to risk assessors and site managers, additional studies would be required to investigate the toxicity of the EM transformation products individually or using chemical mixtures.

Measurement endpoints assessed in this study included adult survival and juvenile production. Study results showed that reproduction was a more sensitive evaluation of effect than adult survival, therefore it should be used to set screening criteria. All ecotoxicological benchmarks determined in this study will be provided to the Ecological Soil Screening Level (Eco-SSLs) workgroup for quality control review by the Eco-SSL task group before inclusion in the Eco-SSL database, and before being used for developing Eco-SSLs for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB for soil invertebrates.

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